Novel poly-uridine insertion in the 3'UTR and E2 amino acid substitutions in a low virulent classical swine fever virus

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Abstract

In this study, we compared the virulence in weaner pigs of the Pinar del Rio isolate and the virulent Margarita strain. The latter caused the Cuban classical swine fever (CSF) outbreak of 1993. Our results showed that the Pinar del Rio virus isolated during an endemic phase is clearly of low virulence. We analysed the complete nucleotide sequence of the Pinar del Rio virus isolated after persistence in newborn piglets, as well as the genome sequence of the inoculum. The consensus genome sequence of the Pinar del Rio virus remained completely unchanged after 28 days of persistent infection in swine. More importantly, a unique poly-uridine tract was discovered in the 3'UTR of the Pinar del Rio virus, which was not found in the Margarita virus or any other known CSFV sequences. Based on RNA secondary structure prediction, the poly-uridine tract results in a long single-stranded intervening sequence (SS) between the stem-loops I and II of the 3'UTR, without major changes in the stem-loop structures when compared to the Margarita virus. The possible implications of this novel insertion on persistence and attenuation remain to be investigated. In addition, comparison of the amino acid sequence of the viral proteins E^{ms}, E1, E2 and p7 of the Margarita and Pinar del Rio viruses showed that all non-conservative amino acid substitutions acquired by the Pinar del Rio isolate clustered in E2, with two of them being located within the B/C domain. Immunisation and cross-neutralisation experiments in pigs and rabbits suggest differences between these two viruses, which may be attributable to the amino acid differences observed in E2. Altogether, these data provide fresh insights into viral molecular features which might be associated with the attenuation and adaptation of CSFV for persistence in the field.

Keywords: CSFV, B/C Domain, E2 glycoprotein, poly-U insertion, secondary structure, amino acid differences, antigenic differences, immunogenicity, pigs, rabbits

1. Introduction

Classical swine fever (CSF) is a highly contagious viral disease affecting domestic pigs and wild boar which results in major losses in stock farming, particularly in developing countries (Moennig et al., 2003). The disease is caused by the CSF virus (CSFV), a member of the genus *Pestivirus* within the family *Flaviviridae* (Simmonds et al., 2012). CSFV comprises three different genotypes 1, 2, and 3, with three to four subgenotypes each (Postel et al., 2013; Beer et al., 2015). The virions consist of a lipid envelope, a capsid, and a single plus-strand RNA genome carrying a single large open reading frame (ORF) flanked by two untranslated regions (UTRs). The ORF encodes a polyprotein of typically 3898 amino acids, which is processed by cellular and viral proteases in the four structural proteins—C, E^{ns}, E1, E2, and in the eight non-structural proteins: N^{pro}; p7; NS2; NS3; NS4A; NS4B; NS5A; and NS5B (Tautz et al., 2015).

The disease is endemic in several South and Central American countries, in the Caribbean region, in Asia, and in some Eastern European countries (Pérez et al., 2012; Postel et al., 2013). Live attenuated CSFV vaccines are highly efficacious, resulting in complete protection from CSF even before neutralising antibodies can be detected, and nearly 100% protection against strains from different genotypes (Aynaud and Launais, 1978; Ganges et al., 2008; Graham et al., 2012). This type of vaccine was applied in numerous countries because of its high efficacy and safety, and the Chinese vaccine strain was the most widely used (C-strain, genotype 1.1) (Liu et al., 2011). Nevertheless, the disease is still endemic in several countries despite intensive vaccination programmes implemented over years. This has been attributed to failures in the responses to vaccination caused by a combination of distinct problems, for example, quality and availability of the vaccine, and gaps in the cold chain between vaccine production and application, among others (Diaz de Arce et al., 2005; Ganges et al., 2008; Shen et al., 2011; Pérez et al., 2012). In endemic areas, a whole range of disease severity from acute haemorrhagic to chronic and subclinical disease has been observed (Van Oirschot et al., 1983; Ganges et al., 2008). A previous study revealed that the increased rate of non-synonymous substitutions in E2 of CSFV among the isolates is associated with this trend (Diaz de Arce et al., 2005). Towards this background, it is worth noting the Cuban CSFV epizootic which started in 1993 after 20 years of epidemiological silence, manifested in several outbreaks each year until today, despite regular vaccination with a lapinised, live, attenuated vaccine C-strain. The molecular

studies within this epidemiological situation showed that viruses currently circulating belong to the 1.4 subgenotype (Postel et al., 2013) and are closely related to those viral strains isolated during the 1993–1997 epizootic, including the CSFV virulent Margarita strain (Díaz de Arce et al., 1999; Díaz de Arce et al., 2005; Ganges et al., 2005; Pérez et al., 2012). Therefore, the most likely origin of the Cuban outbreaks in 1993 was due to the reintroduction of the Margarita strain to the field (Díaz de Arce et al., 1999; Díaz de Arce et al., 2005; Pérez et al., 2012). Similarly, these studies rule out the external reintroduction from another CSFV genogroup.

The role of vaccination in viral evolution has been reported for different diseases affecting both animals and humans. When the immunity is not sterilising, wild strains are able to circulate. Numerous examples of viruses that adapted to this new scenario through immune-escape are available (Cecchinato et al., 2010; Woźniakowski and Samorek-Salamonowicz, 2014; Vera et al., 2015). Recent molecular epidemiology studies from some CSF endemic countries suggest that the viruses circulating in the field have evolved under the positive selection pressure exerted by immune responses to the vaccine, among other factors, and this could favour the generation of new attenuated viral variants that induce milder forms of CSF (Pérez et al., 2012; Ji et al., 2014). The selection pressure analysis previously reported highlighted some viral isolates that showed the G761R replacement into the E2-B/C-domain, which suggests the possible association of this substitution in the virulence and pathogenesis of the virus in the field (Pérez et al., 2012; Ji et al., 2014; Hu et al., 2016). Interestingly, one of these latter Cuban CSFV field isolates, Pinar del Rio (PR-11/10-3), carrying the G761R substitution in the B/C-domain of E2 (Pérez et al., 2012), was able to induce persistence in piglets upon neonatal infection (Muñoz-González et al., 2015a). Therefore, the present study was aimed at determining the virulence and immunogenicity of the Pinar del Rio isolate in comparison to the virulent Margarita strain in weaner pigs. Additionally, E2 sequences and the antigenic reactivity of Pinar del Rio and Margarita were compared. Analysis of the complete nucleotide sequence of Pinar del Rio before and after persistence showed that the consensus genome sequence remained completely unchanged during persistence. Importantly, a novel poly-uridine sequence was discovered in the 3'UTR of the Pinar del Rio isolate.

2. Materials and Methods

2.1. Cells and viruses

We amplified and titrated viral stocks in the porcine kidney cell line PK-15, (ATCC CCL 33) cultured in Dulbecco's modified Eagle medium, (DMEM) supplemented with a 10 % pestivirus-free foetal bovine serum (FBS) at 37 °C in 5 % CO₂. The titrations were performed by end-point dilution using a peroxidase-linked assay, (PLA) and the titres were calculated according to Reed and Muench (Reed and Muench, 1938).

The CSFV Pinar del Rio field isolate originates from the Cuban CSF epizootic of 2010 (PR-11/10-3) (Pérez et al., 2012) and was classified within the new CSFV subgenotype 1.4 (Postel et al., 2013). This virus was isolated by transfection of RNA in PK-15 cells (Meyer et al., 2015) and kept at the European Union Reference Laboratory for Classical Swine Fever (EURL-CSF), in Hannover, Germany, with the reference nomenclature CSF1058. The virulent CSFV Margarita strain which also belongs to subgenotype 1.4 (Díaz de Arce et al., 1999; Postel et al., 2013) was isolated in Cuba in 1958 and has been used since 1965 for vaccine potency testing (Ganges et al., 2005). The CSFV Alfort/187 strain was kindly provided by the CSFV EU Reference Laboratory (EURL), in Hannover, Germany. Finally, the lapinised live attenuated vaccine Labiofam strain belongs to subgenotype 1.1 and has been used in Cuba since 1965 for prophylactic vaccination against CSF (Díaz de Arce et al., 1999; Pérez et al., 2012).

2.2. The experimental infection of the pigs

The pathogenicity of the Pinar del Rio field strain (PR-11/10-3) (Pérez et al., 2012) was evaluated in healthy six-week-old Landrace × Large White × Duroc pigs. The animals were free of porcine circovirus type 2, porcine reproductive and respiratory syndrome virus, and pestiviruses. The animals were distributed in two groups: (1) Pinar del Rio (n=5, numbered 1 to 5) and (2) Margarita (n=3, numbered 6 to 8). Both groups were inoculated intranasally with 2 ml of DMEM containing 10⁵ TCID₅₀ Pinar del Rio field isolate or 10² TCID₅₀ Margarita strain, respectively.

A trained veterinarian recorded the rectal temperature and clinical symptoms of disease daily in a blinded manner. The clinical status of the animals was determined as previously described (Tarradas et al., 2014). The status was scored from 0 to 7 as follows: 0: no signs; 1: mild pyrexia; 2: pyrexia plus mild clinical signs; 3: mild-moderate clinical signs but no nervous disorders; 4: slight nervous disorders and rest of clinical signs moderate; 5: moderate nervous disorders and moderate–severe other clinical signs; 6: severe clinical signs (including nervous disorders); and 7: death. For

ethical reasons, the animals were euthanised either when the clinical score reached 5 or higher, or when they exhibited a fall of the hindquarter, inability to get up to drink, prostration, or when they exhibited moderate nervous disorders. After euthanasia, an exhaustive necropsy was conducted in order to evaluate the presence of pathological symptoms in different organs and tissues. International standards of animal welfare were used, following the regulations for the Institute of Veterinary Medicine (IMV), and Ministry of Agriculture (MINAGRI) of the Republic of Cuba. The protocol was approved by the ethics committee of the MINAGRI of the Republic of Cuba and all efforts were made to minimise any suffering of the animals. The IMV is the official regulatory body of the Republic of Cuba; therefore, additional permits were not required. After the infection, serum samples and nasal and rectal swabs were collected on the first day, then on days 4, 7, 11, 14, 18 and 21 post infection (dpi), and at the time of necropsy (for the Margarita group), or at 21 dpi (for the Pinar del Rio and control groups). Tissue samples of the tonsils, spleen, lymph nodes and ileum were collected. Serum samples were tested with neutralisation peroxidase-linked assay (NPLA) (Terpstra et al., 1984), and the titres obtained were expressed as the reciprocal dilution of serum that neutralised 100 TCID₅₀ of the Alfort 187 strain in 50% of the culture replicates.

2.3. RNA extraction and qRT-PCR for the detection of CSFV RNA

Viral RNA was extracted from 140 µL of the sample by using a QIAamp Viral RNA Mini Kit (Qiagen GmbH) in accordance to the manufacturer's directions. The synthesis of cDNA was performed by random priming, using M-MLV reverse transcriptase as described previously (Díaz de Arce et al., 2009). CSFV RNA in the serum and nasal and rectal swabs, as well as tonsil, spleen, lymph node and ileum samples, was detected using the qRT-PCR assay previously described (Pérez et al., 2011). This test has been used for inter-laboratory comparisons of CSFV diagnoses, organised by the EURL-CSF. Positive results were considered to be threshold cycle values (CT) ≤ 35 . Samples in which fluorescence was undetectable were considered negative. All qRT-PCR analyses were run on a LightCycler 2.0 instrument (Roche Applied Science, Mannheim, Germany).

2.4. Complete genome sequence of the CSFV Pinar del Rio field strain

The complete genome sequence of CSFV Pinar del Rio was determined first, from the original CSF1058 rescued by transfection of RNA in PK-15 cells at EURL-CSF in Hannover, Germany, and second, from the serum of an experimentally and persistently infected piglet collected 28 days post infection with the original CSF1058 stock (pig number seven, Muñoz-González et al., 2015). The total RNA from the lysate CSF1058-infected PK-15 cells and from the serum of the persistently infected pig number seven was prepared using the Viral Amp RNA purification kit (Qiagen) and TRIzol solution (Life Technologies), respectively. To sequence the nucleotides of the original Pinar del Rio CSF1058 viral RNA, Illumina libraries were generated from 15 ng of RNA using a modified protocol of the SCRIPT SEQ version 2 RNA Seq kit (Epicentre Biotechnologies) as described previously (Fischer et al., 2014). The diluted library (2 nM) was sequenced on an Illumina MiSeq instrument (2x250bp paired end; 4,078,934 reads). De novo assembly and taxonomic classification of the assembled contig were performed as described (Becher et al., 2014; Neill et al., 2014).

To determine the complete genome sequence of the CSFV Pinar der Rio recovered from the persistently infected piglet at 28 dpi (Muñoz-González et al., 2015a), we used reverse transcription (RT) with Superscript III reverse transcriptase (Life Technologies) and PCR amplification with Phusion Hot Start II DNA Polymerase (Thermo Scientific) to generate four overlapping long DNA fragments covering the complete genome except the 5' and 3' extremities. The amplicons from four independent RT-PCR assays of each of the long fragments were inserted in pJet1.2 (CloneJET PCR Cloning Kit). The details of the oligonucleotides used for RT-PCR can be obtained on request. The plasmid DNA was sequenced bi-directionally with forward and reverse primers using standard dideoxy-chain terminator sequencing with the BigDye Terminator v3.1 Cycle Sequencing Kit, (Life Technologies) and an Applied Biosystems 3130 Genetic Analyser (Thermo Fisher Scientific). The DNA sequences were assembled with DNA baser software (Heracle BioSoft SRL) using the quality files and default settings to produce a consensus sequence. The 5' and 3' termini of the viral genome were determined using the 5' and 3' RACE System for Rapid Amplification of cDNA Ends (Life Technologies). Twenty-six clones of RACE fragments were sequenced on both strands to establish the consensus.

The nucleotide and deduced amino acid (aa) sequences were analysed with MAFFT and Clustal Omega software (EMBL-EBI). The nucleotide sequence alignment and the

analysis of the Pinar del Rio sequence was performed with other CSFV sequences obtained from GenBank: Alfort/187 (GenBank accession no. X87939), SXCDK (GQ923951), SXYL2006 (GQ122383), RUCSFPLUM (AY578688), BRESCIAAXPLUM (AY578687), NNQianA (AY663656), 96TD (AY554397), 0406CH01TWN (AY568569), GXWZ02 (AY367767), Riems IVI (AY259122), HCLV (AF531433), 39 (AF407339), LPC (AF352565), cF114 (AF333000), Eystrup IVI (AF326963), CS (AF099102), Alfort Tuebingen (J04358), Shimen (AF092448), HCLV (AF091507), Brescia IVI (AF091661), Alfort A19 (U90951), Glentorf (U45478), Riems Giessen (U45477), LOM (EU789580), flcLOM (UE915211), zj0801 (FJ529205), Sp01 (FJ265020), India (EU857642), Thiverval (EU490425), JL1 (EU497410), Shimen HVRI (AY775158), 944IL94TW (AY646427), CHVRI (AY805221), SWH (DQ127910), Brescia (M31768), CAP (X96550), C-strain (AY382481), ALD (D49532), GPE- (D49533), Paderborn (GQ902941), Roesrath (GU233734), Hennef (GU233733), Euskirchen (GU233732), Borken (GU233731), C-ZJ-2008 (HM175885) HEBZ (GU592790) and Koslov (HM237795).

2.5. Nucleotide sequence of the 3'UTR-terminal region of CSFV Margarita and Labiofam strains

To determine the sequence of the 3'UTR-terminal region, we retrieved 15 representative sequences of the three CSFV genotypes, including the vaccine and low and high virulence previously reported CSFV strains, from GenBank. The sequences were aligned using BioEdit (Hall, 1999). Two primers were designed into the 3' end of the CSFV as follows: forward primer (12010-12029), 5'-TCAACATAGTGTTAAGGAGG-3'; reverse primer (12251-12301), 5'-CCGTTAGGAAATTACCTTAG -3'. The nucleotide positions were based on the genome sequence of the Pinar del Rio field strain determined in this study. The one-step RT-PCR protocol was undertaken using the commercially available One-Step RT-PCR Kit (Qiagen). The temperature profile was 30 minutes at 50 °C (reverse transcription), 10 minutes at 95 °C (inactivation reverse transcriptase/activation Taq polymerase), followed by 35 cycles of 30 seconds at 94 °C (denaturation), 35 seconds at 52 °C (annealing) and 30 seconds at 72 °C (elongation). The amplification products were checked by electrophoresis on 2% agarose gel and were directly cleaned with a Wizard® PCR Preps DNA Purification System (Promega, Madison, Wisconsin, USA). The sequencing reactions were conducted under BigDye™ terminator-cycling

conditions using an ABI 3130XL. Forward and reverse sequences obtained from each amplicon were assembled using the Contig Express application in Vector NTI software, version 11 (Invitrogen). Finally, the 3'-UTR RNA secondary structure from the 3'-UTR start nucleotide position (12075) to the genome final (12301) of the CSFV Pinar del Rio field isolate and Margarita strain were predicted using the mfold Web Server software (The RNA Institute college of Arts and Science University at Albany, New York, USA).

2.6. Antibody production and cross-neutralisation assays

Six New Zealand White rabbits weighing 2.5 kg were separated into three groups of two each. Group 1 was immunised with the CSFV Pinar del Rio field isolate derived from the Cuban CSF epizootic of 2010 (PR-11/10-3) (Pérez et al., 2012); group 2 was inoculated with Margarita strain, and group 3 with PBS. The rabbits were subcutaneously immunised three times at 3-week intervals, at six deposits in the back (Huang et al., 2012). The virus solution or PBS was emulsified V/V with Montanide 888 adjuvant (Seppic, France). Each dose of emulsion consisted of $10^{7.2}$ TCID₅₀ of Pinar del Rio or Margarita strains, respectively. Before each inoculation, serum samples were collected from the rabbits via the jugular vein. After euthanising the animals (2 weeks after the last inoculation), sera pools for each inoculated group were prepared. The antibody titres were monitored using the NPLA assay against the homologous and, in parallel, a cross-neutralisation assay against the heterologous virus, respectively (Terpstra et al., 1984). The titres obtained were expressed as the reciprocal dilution of serum that neutralised 100 TCID₅₀ of Pinar del Rio field strain or Margarita strains, respectively in 50% of the culture replicates. Similarly, the neutralising titers were calculated using Kärber's method (Kärber, 1931). Lastly, the mean value of neutralising antibody titres were determined for each virus and experimental group.

In addition, we had previously prepared and stocked pig hyperimmune serum against the CSFV Labiofam vaccine strain. The pig antiserum was generated with two doses at 4-week intervals in 6-week-old CSFV-free domestic pigs. The protocols were approved by the ethics committee of the MINAGRI of the Republic of Cuba. Each dose contained 100 protective doses (PD) administered by intramuscular injection in the neck (Muñoz-González et al., 2015b). The sera were collected at different times post vaccination, and the samples collected 78 days post immunisation were used in the neutralisation test. The titres obtained were expressed as the reciprocal dilution of serum that neutralised

100 TCID₅₀ of Alfort/187 strain, in 50% of the culture replicates. In addition, a neutralisation test was conducted (Terpstra et al., 1984) in order to evaluate the neutralisation capacity of the anti-Labiofam vaccine pig hyperimmune sera against the CSFV Pinar del Rio field isolate originated from the Cuban CSF epizootic of 2010 (PR-11/10-3) (Pérez et al., 2012) and the Margarita strain, respectively. To this end, triplicate heat-inactivated sera with a neutralisation titre of 1:200 against CSFV Alfort/187 strain were mixed with equal volumes of 10^{7.2} TCID₅₀ of Margarita or Pinar del Rio virus suspensions, respectively. The reactions were incubated at 37°C for 1 h and subsequently transferred to 96-well plates with PK-15 cells. Peroxidase-linked assay (PLA) (Wensvoort et al., 1986) was used for viral titration following the statistical methods described by Reed and Muench (Reed and Muench, 1938).

3. Results

3.1. The CSFV Pinar del Rio field isolate is low virulent in weaner pigs

In order to confirm the low pathogenicity of the CSFV Pinar del Rio field isolate that originated from the Cuban CSF epizootic of 2010 (PR-11/10-3) (Pérez et al., 2012) in comparison with Margarita strain, the virulence of these two viruses were assessed side by side in weaner pigs. All the Margarita-infected pigs developed pyrexia (rectal temperature higher than 40 °C) starting at day 4 and lasting until 18 dpi, with peaks greater than 41.5 °C. Moderate and severe clinical symptoms (≥ 4 points in score value), such as anorexia, conjunctivitis, diarrhoea, constipation, abdominal petechiae and nervous disorders, were observed particularly from 12 dpi onwards. Pig number 6 suddenly died at 15 dpi. The other two animals were euthanised for ethical reasons at 17 or 18 dpi (Fig. 1). This contrasted with Pinar del Rio that induced only mild symptoms and slightly elevated body temperatures between 4 and 9 dpi. The clinical scores of the two groups were significantly different from day 6 until the end of the experiment (Fig. 1A). Neutralising antibodies were detected in all the Pinar del Rio-infected pigs as early as 11 dpi and the titres increased up to 800-3200 reciprocal 50% neutralising dilution on 21 dpi, while the titres remained very low in pigs infected with the Margarita strain (Fig. 1B). Compared to Margarita, the replication of Pinar del Rio was delayed by 4 days, and the viral RNA content of the serum and nasal swabs was an average 1000 times lower (Fig. 1C and E). In the rectal swabs, the viral RNA content was low for both viruses and the differences were less pronounced (Fig. 1D). A clearly lower viral RNA content of the same order of magnitude was also detected in the tissue samples,

that is, the tonsils, spleen, lymph nodes and ileum of Pinar del Rio- versus Margarita-infected pigs (Fig. 1F).

3.2. Complete nucleotide sequence analysis of CSFV Pinar del Rio revealed a novel poly-uridine tract in the 3'UTR

In order to determine whether the Pinar del Rio virus did change genetically during its persistence in piglets, the complete genome sequence of the virus was determined from serum collected after 28 days of persistence and from the original Pinar del Rio virus stock (CSF1058). The complete sequence of the Pinar del Rio virus was determined from both the inoculum and the virus recovered from the persistently infected piglets. However, 11 nucleotides from the 5' end and 53 from the 3' end were not possible to sequence from the Pinar del Rio inoculum by the mass sequencing Illumina method. Eventually, the 3' end was fully amplified and sequenced by using the Sanger Methods as previously described. Remarkably, the two consensus nucleotide sequences from the Pinar del Rio virus stock (CSF1058) and the Pinar del Rio virus collected after 28 days of persistence in a pig were 100% identical. This sequence, the first obtained from the CSFV 1.4 genogroup, was deposited in GenBank with the accession number KX576461.

The complete 3'-UTR sequences of the Margarita and Labiofam strains were also determined and deposited in GenBank with the accession number (LT601427) and (LT601428), respectively. All the Pinar del Rio sequences analysed showed a novel uninterrupted polyuridine (poly-U) sequence of an average length of 36 uridines in the 3'-UTR starting at nucleotide 12225, a position where all other CSFV strains sequenced to date including the Margarita strain have typically only 4 to 5 uridines (Fig. 2). This poly-U sequence was also found in the Pinar del Rio virus stock used to inoculate the newborn piglets. The length of the poly-U sequence was heterogeneous, varying from 19 to 47 nucleotides in a total of 26 independent cDNA clones analysed. In some individual clones, the poly-U was interrupted with one or two cytidines and/or adenosines. It is worth noting that in the Labiofam vaccine strain, a poly-U tract was found starting at position 12137 within the 3'UTR, that is, nearly 90 nucleotides upstream of the position of the poly-U stretch discovered in the genome of Pinar del Rio (Fig. 2).

3.3. The secondary structure prediction of the 3'-UTR end

The effect of the novel poly-U sequence on the predicted RNA secondary structure was compared in the Pinar del Rio and Margarita strains using the mFold program. A secondary structure containing four stem-loops (SL-I to SL-IV) was obtained with the 3'-UTR of both CSFV Margarita and Pinar del Rio (Fig. 3). The poly-U insertion in Pinar del Rio forms a long single-stranded intervening sequence (SS) that increases the distance between SL-I and SL-II. The 3'-UTR of CSFV Pinar del Rio and Margarita strain have the same predicted RNA secondary structure when the poly-U sequence of Pinar del Rio is removed.

3.4. The amino acid differences in the envelope proteins of the CSFV Pinar del Rio and Margarita virus clusters in E2

The envelope protein sequences of E^{ms}, E1, E2 and p7 were compared by aligning the sequence data available for CSFV Pinar del Rio (Genbank accession nos. JX028204 and KX576461) and Margarita (Genbank accession nos. JX028201 and AJ704817.1). Three conservative K385R, V674I, and D1097E substitutions were found in E^{ms}, E1 and p7, respectively. Six substitutions were found in E2, two of them, G761R and L763S, being located within the B/C domain (Fig 4). The four other changes were the conservative I780V and the non-conservative D898V, L956S, and T1028A mutations. Of note, a single non-synonymous change was found between the newly determined Pinar del Rio sequence (KX576461) and the partial sequence of Pinar del Rio published previously (JX028204), resulting in a H852Y substitution in E2 of the newly determined Pinar del Rio sequence (KX576461). At this position, the Margarita virus carries H852 (Fig. 4) as Pinar del Rio JX028204.

3.5. Differences in the antigenic reactivity and immunogenicity between Pinar del Rio and Margarita strains

In order to determine whether the amino acid differences found in E2 modify the antigenic properties of Pinar del Rio and Margarita, we first compared the susceptibility of the two viruses to neutralisation by the porcine hyperimmune serum against the CSFV Labiofam vaccine strain. A porcine hyperimmune serum against Labiofam vaccine strain neutralised the Margarita virus completely from 10^7 TCID₅₀/mL to undetectable levels, while the infectivity of the Pinar del Rio virus was reduced by three log₁₀ only, from $10^{7.2}$ TCID₅₀/mL to $10^{4.3}$ TCID₅₀/mL.

Finally, we compared the immunogenicity of Pinar del Rio and Margarita in rabbits. Rabbits were used for this purpose because the virulent Margarita strain causes severe clinical disease and even death in infected pigs before the induction of an effective humoral response. Neutralising antibody titres were determined against both the homologous and the heterologous virus in a cross-neutralisation assay. Higher neutralising antibody titres were found in the Margarita-immunised rabbits with titres of 7499 and 2239 against the homologous and heterologous viruses, respectively. In contrast, 13 and 8 times lower neutralisation titres were detected in sera from the Pinar del Rio-immunised animals, with titres of 562 and 282 against the homologous (Pinar del Rio field isolate) and heterologous (Margarita strain) virus strains, respectively.

4. Discussion

Despite the importance of low virulent CSFV strains, available data on the molecular biology of CSFV attenuation and pathogenesis induced by this type of viral strain are scarce. This study describes the full-length nucleotide sequence of the Pinar del Rio isolate PR-11/10-3 (Pérez et al., 2012). This field strain was recovered in the west of Cuba in 2010 with the G761R replacement into the E2-B/C-domain, being previously suggested its implication in the virulence and the pathogenesis of CSFV in the field (Shen et al., 2011; Pérez et al., 2012; Hu et al., 2016). Accordingly, molecular and antigenic properties of the E2 protein from Pinar del Rio virus and the virulent Margarita strain, the most likely origin for the Cuban outbreaks in 1993, were compared. The virulence of the Pinar del Rio isolate and Margarita strain was also evaluated side by side in weaner pigs. In accordance with previous data (Postel et al., 2015), the field isolate Pinar del Rio strain induced only very mild clinical signs in the infected piglets, along with only short-term low viral RNA levels in the blood, organs and body secretions. Considering the level of CSFV replication *in vivo* as a major correlate of virulence (Leifer et al., 2013; von Rosen et al., 2013; Tamura et al., 2014; Tarradas et al., 2014), these data confirmed the low virulence of the Pinar del Rio field isolate, being in line with previous studies from endemic countries (Pérez et al., 2012; Ji et al., 2014; Hu et al., 2016).

During experimental infection, neutralising antibody responses were mainly detected in the Pinar del Rio-infected piglets, while the virulent Margarita strain was unable to induce an effective humoral response. This confirmed the ineffectiveness of the virulent CSFV strains to induce neutralising antibodies during severe CSF disease (Ganges et

al., 2005 and Tarradas et al., 2010). Pigs with higher neutralising antibody titres had lower CSFV RNA loads, especially at 21 dpi when two out of five pigs had cleared the virus. Nevertheless, viral RNA was detected in 55% of the tissues samples at necropsy, principally in the tonsils and spleen of the Pinar del Rio-infected pigs (Fig. 1F), corroborating the role of these lymphoid organs in the spread and persistence of CSFV (Ganges et al., 2008; Tarradas et al., 2014; Muñoz-González et al., 2015a).

We have previously reported that the early post-natal infection of piglets with the Pinar del Rio isolate resulted in persistent infection lasting for at least 4 weeks in the absence of any detectable antibody response (Muñoz-González et al., 2015a). In this study, we determined the complete genome sequence of the Pinar del Rio virus used to infect the newborn piglets and the virus re-isolated from the serum after 28 days of persistence. Importantly, this full-length consensus sequence represents the first complete genome sequence of a CSFV subgenotype 1.4. It is noteworthy that the consensus sequences obtained before and after persistence were completely identical. As a result, the CSFV genome sequence of low virulent Pinar del Rio field strain remains unaltered after 28 days of persistent infection in piglets (Muñoz-González et al., 2015a). It was recently determined that viral samples from pigs infected with highly virulent CSFV isolates “Koslov” or “Brescia” showed higher quasispecies diversity and more nucleotide variability, compared to samples of pigs infected with low and moderately virulent isolates (Töpfer et al., 2013). In addition, a previous work analysing the Cuban CSFV population dynamics should be noted. This study revealed a decrease in the genetic diversity of the viral population from the epizootic beginning, when the acute disease was prevalent, to recent years (Pérez et al., 2012). The high stability showed by this low virulent CSFV field strain may be linked to a potential adaptive advantage, which may favour the prevalence of the low virulent strain circulating in the field. Considering the role of the mechanism showing the suppression of superinfection (SIE) in CSFV persistently infected pigs, the possible outcome of the generation of the new low pathogenicity CSFV strains circulating in an endemic situation and the impact of the SIE on disease control cannot be underrated (Muñoz-González et al., 2016).

A unique uninterrupted poly-U insertion of an average length of 36 nucleotides was found in the 3'-UTR the Pinar del Rio genome. Such a poly-U insertion has never been described before at this position in the 3'UTR of pestiviruses according to GenBank. Also of interest is a similar 6- to 32-nucleotide-long U-rich insertion that can be found

in the 3'-UTR of several attenuated CSF vaccine virus strains, such as HCLV, C-strain, Porcivac, Rovac, Russian LK and Thiverval, nearly 90 nucleotides upstream compared to the Pinar del Rio poly-U (Wu et al., 2001; Fan et al., 2008). This latter U-rich sequence was also found in the Labiofam vaccine strain used in Cuba to control CSFV over decades, as well as and in the lapinised Harbin vaccine strain (Genbank accession number AY805221.1) (Fig. 2). Although the possible functions of the U-rich insertions in the 3'-UTR of the attenuated vaccine strains are still unknown, it has been speculated that the U-rich sequence in the C-strain vaccine was acquired during the adaptation of the virus to the rabbit host (Li et al., 2014). Accordingly, it is of interest to speculate that the U-rich sequences of the vaccine strains and the poly-U insertion in the genome of Pinar del Rio contribute to attenuation. Of note, the long poly-U/UC tract located in the 3'UTR of HCV was found to trigger innate immune induction via RIG-I, which was dependent on the composition and length of the poly-U stretch (Schnell et al., 2012; Kell et al., 2015). The 3'-UTR of pestiviruses contains *cis*-active elements that are indispensable for viral replication and translation (Austermann-Busch and Becher, 2012; Li et al., 2014). This structure also plays a regulatory role in IRES-mediated translation (Huang et al., 2012). Previous studies showed that the insertion and deletion of nucleotides in the 3'-UTR of pestiviruses may lead to significant changes in viral RNA synthesis (Xiao et al., 2004; Pankraz et al., 2005). Although the implication and functionality of the poly-U insertion in the Pinar del Rio field strain remain to be determined, it could be associated with decreased virulence and a resulting potential advantage of this viral strain within the endemic situation which may favour the establishment of low virulent CSFV. Previously described RNA secondary structure models of CSFV 3'-UTR predict four consecutive stem-loop structures, SL-I, SL-II, SL-III and SL-IV (Fan et al., 2008; Huang et al., 2012). These can also be predicted for the Margarita strain. The poly-U insertion in the genome of Pinar del Rio leads to a long single-stranded intervening sequence (SS) between SL-I and SL-II in the predicted RNA secondary structure (Fig 3). Previous studies revealed that the SL-I and the SS region between SL-I and SL-II are essential for viral replication (Pankraz et al., 2005; Huang et al., 2012). Pestivirus replication depends on the binding of microRNA-17 (miR-17) to a highly conserved sequence between SL-I and SL-II (Scheel et al., 2016). This miR-17 binding site is located 3 nucleotides downstream of the large poly-U insertion identified in the Pinar del Rio genome. The binding of miR-17 by the

pestivirus genome enhances translation and RNA stability. Whether and how the poly-U stretch and the miR-17 binding may be linked functionally is a matter of future studies.

In our comparison of the sequences of the Pinar del Rio and Margarita strains, we also noticed the fact that the amino acid differences in the envelope proteins clustered within E2. Among these was the previously described G761R mutation within the B/C domain of E2 (Shen et al., 2011; Pérez et al., 2012; Hu et al., 2016). Previous studies have suggested that the TAVSPTTLR motif and an additional 13 amino acids in the carboxy-terminal part of E2 may be significantly implicated in CSFV virulence (Risatti et al., 2006; Risatti et al., 2007; Shen et al., 2011; Tamura et al., 2012). However, these amino acid residues are conserved in Margarita and Pinar del Rio. In the p7 protein, which is a viroporin possibly implicated in CSFV virulence (Gladue et al., 2012), a conservative D (Margarita) to E (Pinar del Rio) substitution was found at position 1096. This position connects two transmembrane helices of p7. For the related hepatitis C virus (HCV) it has been reported that changes around this site (aa 1095 to 1101) prevent HCV replication in chimpanzees (Sakai et al., 2003).

Finally, we found differences in the antigenic properties of E2 of the Pinar del Rio and Margarita virus. The sera from pigs vaccinated with the Labiofam vaccine were less efficient at completely neutralising the Pinar del Rio field strain than the Margarita strain. In addition, when using sera from rabbits infected with either of the two viruses, we found that the neutralising antibody titres against both the homologous and heterologous viruses were considerably lower in the Pinar del Rio-infected rabbits compared to rabbits infected with Margarita. Glycosylation of viral envelope proteins may influence the immunogenicity and the sensitivity of the virus to neutralising antibodies by maintaining the appropriate conformation of proteins (Li et al., 2008). Accordingly, mutations in the E2 B/C domain including the amino acid residues 761 and 763 might lead to variations in virus neutralisation (Chen et al., 2010; Chang et al., 2012; Hu et al., 2016). Additionally, previous studies showed that alteration into the B/C domain, mainly from amino acids 690 to 773, reduced the binding of pig serum raised against the C strain (Tong et al., 2015). Thus, the changes found in E2 of the attenuated CSFV strain Pinar del Rio may be associated with a possible viral escape from neutralising antibodies, which may be related to the viral persistence and low virulence of Pinar del Rio.

In conclusion, the evolutionarily related low and high virulent CSFV strains of Pinar del Rio and Margarita represent a valuable virus pair for studying CSFV virulence factors. Future experimental approaches employing reverse genetics are required to clarify the issues identified in the present study.

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Competing interests

The authors declare that they have no competing interests.

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Figure Legends

Figure 1. Assessment of the virulence of the CSFV strains Pinar del Rio and Margarita in weaner pigs. The clinical scores (A), neutralising antibody titres (B), and viral RNA load in nasal swabs (C), rectal swabs (D) and serum (E) were monitored at the indicated days post infection (dpi) for the pigs infected with CSFV Pinar del Rio (grey lines and symbols, pigs # 1 to 5) or Margarita (black lines and symbols, pigs # 6 to 8). For the clinical scores, the mean values are shown with error bars representing the standard deviation. At necropsy, the viral RNA load was determined in the tonsils, spleen, lymph node and ileum (F). Viral RNA was quantified by qRT-PCR (B to F) and represented with the Ct values. Ct values equal to (dotted line) or smaller than 35 were considered positive. *Pig number 6 died at 15 dpi and it was not possible to collect tissue samples from this animal.

Figure 2. Comparison of the 3'UTR sequences of the CSFV Pinar del Rio isolate, Margarita strain, the Labiofam vaccine strain and other selected strains. The 3'UTRs were aligned from nucleotide position 12074 of Pinar del Rio to the 3' end. The Genbank accession numbers are indicated with the virus nomenclature, and the poly-uridine tracts are boxed.

Figure 3. Secondary structure prediction of the 3'-UTR of the CSFV Pinar del Rio and Margarita strains. The basic profile contains SL-I, SL-II, SL-III, SL-IV and region SS. (A) Secondary structure prediction of the 3'-UTR of CSFV Margarita strain 226 bp, free energy = -51.29. (B) Secondary structure prediction of the 3'-UTR of CSFV Pinar del Rio field strain 257 bp, free energy = -53.59.

Figure 4. Amino acid sequence alignment of the glycoprotein E2 region. The last 23 residues of E1, the complete E2, and 47 first residues of p7 of CSFV Margarita (AJ704817) and Pinar del Rio (KX576461) are shown. The amino acid differences between the two strains are highlighted in boldface. Arrows indicate the first amino acid of E2 and p7.

A

B

C

D

E

F

A

B

E1

E2

Margarita
PdR

IKVLRGQ**V**VQGVIIWLLLVTGAQGR**L**ACKEDFRYAISSTNEIGLLGA**E**GLTTT**W**KDYDHNL
IKVLRGQ**I**VQGVIIWLLLVTGAQGR**L**ACKEDFRYAISSTNEIGLLGA**E**GLTTT**W**KDYDHNL
*****:*****

Margarita
PdR

QLDDGT**I**KAICTAGSFKV**I**ALNVVSRRYLASLHK**G**AL**P**TSVTFELLFDGTSPS**I**EEMGDD
QLDDGT**I**KAICTAGSFKV**I**ALNVVSRRYLASLHK**R**AS**P**TSVTFELLFDGTSPS**V**EEMGDD
***** * *****:*****

Margarita
PdR

FGFGLCPFDTS**P**VVKGRYNTTLLNGSAFYLVCP**I**GW**T**G**V**IECTAVSPTTLRTEV**V**KTFRR
FGFGLCPFDTS**P**VVKGRYNTTLLNGSAFYLVCP**I**GW**T**G**V**IECTAVSPTTLRTEV**V**KTFRR
*****

Margarita
PdR

E**K**FP**F**H**R**KDCVTTT**V**ENEDLFYCRLGGNW**T**CVKG**E**PV**I**YTGG**L**V**K**QCR**W**CG**F**D**F**NEPDGL
E**K**FP**F****Y**RKDCVTTT**V**ENEDLFYCRLGGNW**T**CVKG**E**PV**I**YTGG**L**V**K**QCR**W**CG**F****V**FNEPDGL
*****:*****

Margarita
PdR

PHYPIGKCILANETGYR**I**VDSTDCNRNGVVISTEGSHECL**I**GN**T**SVKV**H**ALDERLG**P**MP**C**
PHYPIGKCILANETGYR**I**VDSTDCNRNGVVISTEGSHECL**I**GN**T**SVKV**H**ASDERLG**P**MP**C**
*****

Margarita
PdR

RPKEIVSSEGPVR**K**T**S**CTFNYTKTLRNKY**E**PRDSY**F**Q**Q**Y**M**LKGEY**Q**Y**W**FDLDVTDH**H**SD
RPKEIVSSEGPVR**K**T**S**CTFNYTKTLRNKY**E**PRDSY**F**Q**Q**Y**M**LKGEY**Q**Y**W**FDLDVTDH**H**SD
*****

P7

Margarita
PdR

Y**F**TEFLVLVVALLGGRYVLWLIVTYV**V**LTEQLAAG**L**QLG**Q**GEVVL**I**GN**L**ITHTDIEV**V**
Y**F****A**EFLVLVVALLGGRYVLWLIVTYV**V**LTEQLAAG**L**QLG**Q**GEVVL**I**GN**L**ITHTDIEV**V**
:***

Margarita
PdR

YFLLLYLIMR**D**PIKKWILL**L**FFH
YFLLLYLIMR**E**PIKKWILL**L**FFH
*****:*****